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Quantitative multiple reaction monitoring proteomic analysis of G β and G γ subunits in C57Bl6/J brain synaptosomes

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Abstract

G $\beta\gamma$ dimers are one of the essential signaling units of activated G protein coupled receptors (GPCRs). There are five G β and twelve G γ subunits in humans; numerous studies have demonstrated that different G β and G γ subunits selectively interact to form unique G $\beta\gamma$ dimers, which in turn may target specific receptors and effectors. Perturbation of G $\beta\gamma$ signaling can lead to impaired physiological responses. Moreover, previous targeted multiple reaction monitoring (MRM) studies of G β and G γ subunits have shown distinct regional and subcellular localization patterns in four brain regions. Nevertheless, no studies have quantified and compared their individual protein levels. In this study, we have developed a quantitative MRM method to not only quantify but also compare the protein abundance of neuronal G β and G γ subunits. In whole and fractionated crude synaptosomes, we were able to identify the most abundant neuronal G β and G γ subunits and their subcellular localizations. For example, G β_1 was mostly localized at the membrane while G β_2 was evenly distributed throughout synaptosomal fractions. The protein expression levels and subcellular localizations of G β and G γ subunits may affect the G $\beta\gamma$ dimerization and G $\beta\gamma$ -effector interactions. This study offers not only a new tool to quantify and compare G β and G γ subunits, but also new insights into the *in vivo* distribution of G β and G γ subunits, and G $\beta\gamma$ dimer assembly in normal brain function.

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INTRODUCTION

G protein-coupled receptors (GPCRs) are the largest and most diverse group of membrane proteins, encoded by approximately 800 genes in the human genome, and are some of the most successful drug targets^{1, 2}. Upon activation, GPCRs transduce extracellular signals into various intracellular responses by the activation and dissociation of heterotrimeric G proteins. Heterotrimeric G proteins, made up of $G\alpha$, $G\beta$, and $G\gamma$ subunits, dissociate after activation of GPCRs into a GTP-bound $G\alpha$ ($GTP-G\alpha$) and a $G\beta\gamma$ dimer; each signals to a number of effectors. $G\beta\gamma$ dimers interact with adenylyl cyclases and phospholipase $C\beta$, in addition to PI3-kinase and components of the mitogen-activated protein kinase cascade^{3–8}. In the central nervous system (CNS), $G\beta\gamma$ dimers also interact with voltage-dependent calcium (VDCC) and inward-rectifying potassium (GIRK) channels, and soluble NSF attachment proteins (SNARE) to regulate neurotransmitter release at the synapse^{9–16}. Although many of these $G\beta\gamma$ -effector interactions and downstream signaling cascades are well understood, it is still unclear which combination of $G\beta\gamma$ dimers are present *in vivo* and what factors control the specificity of $G\beta\gamma$ dimers to their effectors. Given the diversity of GPCRs, G proteins, and $G\beta\gamma$ effectors, and the importance of GPCRs as drug targets, examining the expression and subcellular localization of $G\beta$ and $G\gamma$ subunits will aid in our understanding of not only the regulatory effects of $G\beta\gamma$ dimer specificity in physiology but also in disease pathophysiology such as depression, ADHD, and Parkinson's disease¹⁷.

In mammals, there are five different $G\beta$ genes and twelve different $G\gamma$ genes encoding each subunit^{18–20}. $G\beta_{1–4}$ share up to 90% amino acid sequence identity whereas $G\beta_5$ is only 50% identical^{21, 22}. In contrast, $G\gamma$ subunits are very divergent, sharing only 30–70% sequence identity^{21, 22}. Made up of two α -helices, $G\gamma$ subunits can be post-translationally modified at the processed C-terminal cysteine which is carboxymethylated and modified with a farnesyl or geranylgeranyl moiety via a thioether bond. These modifications aid $G\beta\gamma$ dimers in membrane localization^{23, 24}. Together, $G\beta$ and $G\gamma$ subunits form $G\beta\gamma$ dimers and once assembled, act as signaling units for GPCRs. Although we do not fully understand the selectivity of $G\beta\gamma$ dimers for various GPCRs and effectors, some studies have hypothesized that $G\beta$ subunits determine the $G\beta\gamma$ -effector specificity, while $G\gamma$ subunits confer $G\beta\gamma$ -receptor specificity^{3, 22, 25–31}. To date, numerous genetic deletion studies and knockout animal studies have suggested specific roles for different $G\beta$ and $G\gamma$ subunits in intact cells and mice^{30, 32}. Various $G\beta$ and $G\gamma$ subunits are implicated in neurodevelopmental disability, hypotonia, and seizures^{33–35}. These unique physiological phenotypes of each $G\beta$ and $G\gamma$ subunits suggest a great deal of specificity in $G\beta\gamma$ dimerization and signaling^{36–38}. *In situ* hybridization studies of $G\beta$ and $G\gamma$ subunits in the CNS indicate how the distribution of $G\beta$ and $G\gamma$ subunits may affect $G\beta\gamma$ dimerization in specific brain regions and cell types^{7, 21, 22}. Some $G\beta$ and $G\gamma$ subunits are ubiquitously expressed, whereas others are localized in specific brain regions and cell types^{21, 39–43}. Because most cell types express multiple $G\beta$ and $G\gamma$ subunits, specific expression levels and localization may influence intracellular signaling cascades through the formation of specific $G\beta\gamma$ dimers.

Although there are 60 different theoretical combinations of $G\beta\gamma$ dimers^{44, 45}, numerous *in vitro* assays and yeast-two hybrid analyses have indicated that not all theoretical $G\beta\gamma$ dimers exist, are equally expressed, or interact with $G\alpha$ subunits, receptors, effectors, and

downstream signaling factors^{19, 32, 35, 46–52}. Each G β and G γ subunit shows widely varying affinities for one another^{19, 22, 53}. While G β_1 and G β_4 dimerize with all G γ subunits, G β_2 and G β_3 are unable to dimerize with G γ_1 and G γ_{1154} . G $\beta_2\gamma_1$ shows a stronger association than G $\beta_2\gamma_4$ ^{22, 34, 55}. Different affinities between G β and G γ subunits, in combination with the expression and localization of individual G β and G γ subunit, may determine which G $\beta\gamma$ dimers are active in a given cell²¹. Interestingly, various G $\beta\gamma$ dimers have been reported to have different affinities to their effectors. G $\beta_1\gamma_2$ has a 40-fold higher affinity for SNARE and a 20-fold higher inhibition of exocytosis than G $\beta_1\gamma_1$ ⁵⁶. We speculate that the difference in the G γ subunit, per se, or in the post-translation modification of G γ_1 and G γ_2 may cause the change in affinity. Such expression and affinity diversity of G β and G γ subunits and the affinity of G $\beta\gamma$ -effector interactions may also suggest that specific dimers could permit specialized roles in signal transduction pathways through association with particular GPCRs. For example, G $\beta_2\gamma$ and G $\beta_4\gamma$ dimers may specifically interact with adrenergic and opioid GPCRs while G $\beta_1\gamma$ and G $\beta_3\gamma$ dimers, particularly G $\beta_1\gamma_3$ and G $\beta_3\gamma_4$, may preferentially couple with somatostatin and muscarinic M4 GPCRs^{57–59}. Thus, a greater understanding of the expression and subcellular localization of G β and G γ subunits in the CNS will be particularly important in determining the physiologically relevant G $\beta\gamma$ dimers, the roles of each unique G $\beta\gamma$ dimer in regulating signaling cascades, and their impact in neurological diseases and GPCR targeted drug mechanisms.

Despite many attempts to quantify G β and G γ protein level^{39, 42, 43, 60–62}, it has been difficult to develop reliable subunit-specific detection methods due to the high sequence homology between subunits and lack of subunit-specific antibodies⁶³. To overcome this issue, we previously developed a targeted multiple reaction monitoring (MRM)^{64, 65} mass spectrometric approach to identify neuronal G β and G γ subunits. We found a regional and subcellular expression pattern of four G β (G β_1 , G β_2 , G β_4 , and G β_5) and six G γ (G γ_2 , G γ_3 , G γ_4 , G γ_7 , G γ_{12} , and G γ_{13}) subunits in mouse crude synaptosomes of cortex, cerebellum, hippocampus, and striatum⁶². However, we were unable to quantify and compare the expression level of each G β and G γ subunit. Here, we use the quantitative MRM method of G β and G γ subunits, in combination with the isotopic labeling of standards and Skyline analysis^{62, 66}, to generate a comprehensive, quantitative brain map of G β and G γ subunits. We measured and compared the protein level and subcellular localization of neuronal G β and G γ subunits and predicted the *in vivo* expression level of G $\beta\gamma$ dimers, further supporting the G $\beta\gamma$ specificity to particular GPCRs and effector proteins to maintain normal brain function.

MATERIALS AND METHODS

Synaptosome preparation

All animal handling and procedures were conducted in accordance with the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Vanderbilt Institutional Animal Care and Use Committee. Crude synaptosomes were made from adult, male C57Bl6/J mice as described previously^{62, 67, 68}. Briefly, whole brains were homogenized in 20 mL of a 0.32 M sucrose solution (0.32M sucrose, 4.2 mM HEPES pH 7.4, 0.1 mM CaCl₂, 1 mM MgCl₂, 1.54 μ M aprotinin, 10.7 μ M leupeptin, 0.95 μ M pepstatin,

and 200 μ M PMSF). Homogenates were centrifuged at 1000 $\times$ g and 4°C for 10 min and supernatants containing synaptosomes (S1) were transferred to clean conical tubes. Pellets were resuspended in 20 mL of 0.32 M sucrose solution and centrifuged again. Pellets were discarded. Supernatants (S1) were combined and centrifuged at 10,000g and 4°C for 20 min to produce the crude synaptosome pellet. Crude synaptosomes were stored at -80°C.

Synaptosome lysate

Crude synaptosomes were gently resuspended in 4 mL of RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 1 mM EDTA, 1.54 μ M aprotinin, 10.7 μ M leupeptin, 0.948 μ M pepstatin, and 200 μ M PMSF) using a 25 gauge needle to lyse membranes. Lysate concentrations were determined with a BCA assay kit (Pierce) and diluted to 1 mg/mL using RIPA buffer. Diluted homogenate was placed on a rotator for 1 hr and maintained at 4°C. Homogenates were transferred to 2 mL Eppendorf and centrifuged at 14,000 rpm and 4°C for 10 min to separate the triton-soluble and insoluble fractions. Supernatants, the triton-soluble fractions, were collected. Protein concentrations were determined with a BCA assay kit (Pierce).

Subcellular fractionation

Subcellular fractions were prepared as previously described^{62, 69} (Fig. 4). Briefly, crude synaptosomes were gently resuspended in 4 mL of hypotonic lysis buffer (20 mM Tris pH 6.0, 0.1 mM CaCl₂, 1 mM MgCl₂, 1% Triton X-100, 1.54 μ M aprotinin, 10.7 μ M leupeptin, 0.95 μ M pepstatin, and 200 μ M PMSF) and incubated on ice for 20 min to lyse membranes. Lysates were subjected to ultracentrifugation at 100,000 $\times$ g and 4°C for 2 hrs using a SW-55 Ti rotor (Beckman Coulter) to separate supernatants consisting of the synaptosomal cytosolic fractions from membrane fractions. Synaptosomal cytosolic fraction may contain crude vesicles⁷⁰. Supernatants were transferred to clean conical tubes. Pellets contacting membrane fractions were resuspended in 2 mL of Tris pH8.0 buffer (20 mM Tris pH 8.0, 1% Triton X-100, 1.54 μ M aprotinin, 10.7 μ M leupeptin, 0.95 μ M pepstatin, and 200 μ M PMSF) and incubated on ice for 20 min. Lysates were centrifuged at 10,000 $\times$ g and 4°C for 30 min and supernatants containing enriched presynaptic fractions were collected. Finally, pellets were resuspended in 400 μ L of a 1 $\times$ PBS/ 0.5% SDS buffer and centrifuged at 10,000 $\times$ g and 4°C for 30 min. Supernatants containing enriched postsynaptic fractions were collected. Pellets were discarded. Protein concentrations of each fraction were determined with a BCA assay kit (Pierce).

Antibodies

Mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Millipore, MAB374, 1:20,000), mouse anti-SNAP25 (Santa Cruz, sc-376713, 1:500), mouse anti-syntaxin-1 (Santa Cruz, sc-12736, 1:2,000), mouse anti-N-methyl-D-aspartate receptor-1 (NMDAR1) (BD Pharmingen, 556308, 1:2,000), mouse anti-postsynaptic density-95 (PSD-95) (Neuromab, 75-028, 1:20,000), and rabbit anti-G β (Santa Cruz, sc-378, 1:15,000) were used. HRP-conjugated secondary antibodies were obtained from Perkin-Elmer and Jackson ImmunoResearch and used at the following dilutions: goat anti-rabbit (1:20000), goat anti-mouse (1:10,000 for NMDAR1 and syntaxin, and 1:20,000 for PDS-95, GAPDH, and SNAP25) and mouse anti-rabbit light chain specific 1:7,500 (G β).

Immunoblot Analysis

To examine the fractionation of crude synaptosomes, western blot analysis was performed on 7 μ g of synaptosomal cytosolic, presynaptic, and postsynaptic fractions as described⁶².

Protein purification of G β γ dimers

G $\beta_1\gamma_1$ was purified from the bovine retina as described⁷¹. Recombinant His₆-tagged G $\beta_5\gamma_2$ were expressed in Sf9 cells and purified using nickel-nitrilotriacetic acid affinity chromatography (Sigma-100 Aldrich, St. Louis, MO). Human G $\beta_1\gamma_2$, containing an N-terminally hexahistidine tagged β subunit, was expressed in High Five cells using a dual promoter insect cell expression vector described previously⁷². G $\beta_1\gamma_2$ was purified from membrane extracts of High Five cells harvested 48 hrs post infection using nickel-nitrilotriacetic acid affinity, and anion exchange chromatography as described previously⁷³. Fractions containing G $\beta_1\gamma_2$ were subsequently pooled and buffer exchanged into 20 mM HEPES pH 8.0, 100 mM NaCl, 0.5 mM EDTA, 2 mM MgCl₂ and 1 mM DTT using a S200 column. G $\beta_1\gamma_2$ fractions were then concentrated to 5 mg/mL, as determined by Bradford analysis, in a 30kD cut-off Amicon Ultra-15 Centrifugal Filter Unit, flash frozen in liquid nitrogen, and stored at -80°C until future use.

Heavy labeled proteotypic peptides detection analysis

To examine the detection of each heavy labeled proteotypic peptide, we selected one heavy labeled peptide with the largest area under the curve from Betke et al.⁶² study (Bold in Table 1), for each G β and G γ subunit (Table 1). 0.04, 0.2, 0.4, 4, 20, 40, 100 fmol of each heavy labeled peptide were pooled and mixed with bovine serum albumin (BSA), analyzed by TSQ Vantage triple quadrupole mass spectrometry (Thermo Scientific), and quantified using Skyline⁶⁶ (data not shown).

Heavy labeled peptide cocktail

Based on the amino acid sequence of tryptic digested proteins and fragment ion signal intensities in the previous study⁶², we have selected two proteotypic peptides for four G β (G β_1 , G β_2 , G β_4 , and G β_5) and six G γ (G γ_2 , G γ_3 , G γ_4 , G γ_7 , G γ_{12} , and G γ_{13}) subunits detected in crude synaptosomes. Proteotypic peptides listed in Table 1 were synthesized via SPOT synthesis (JPT Peptide Technologies). The arginine or lysine at the C-terminal of these peptides was isotopically labeled with heavy ¹³C or ¹⁵N with a trypsin cleavable Qtag to quantify (JPT). Heavy labeled peptides are 8–10 Da heavier without changing the physiological properties and chemical reactivity compared to the non-labeled proteolytic peptides. One nmol of each peptide was resolubilized in 200 μ L HPLC grade water, 200 μ L of 10% acetonitriles to make a stock concentration of 5 pM/ μ L. According to the mass spectrometry signal strength of each proteotypic peptide, different amount of peptides were pooled to create a “heavy labeled peptide cocktail.” The cocktails were mixed with BSA, 1.5 M Tris, and HPLC grade water before being reduced with 2.5 mM TCEP for 30 min and alkylated with 5 mM iodoacetamide for 30 min in the dark. The cocktail was then digested overnight with 0.5 mg of trypsin then stored at -80°C . Before each MRM analysis, the cocktail was acidified with 1.5 μ L formic acid and added to samples as internal standards for quantification.

Quantitative MRM of G β and G γ subunits

As shown in Fig. 2, samples containing G β and G γ subunits were separated by 12.5% acrylamide, SDS-PAGE and stained with colloidal Coomassie Blue (Invitrogen). Using G $\beta_1\gamma_1$ and G $\beta_5\gamma_2$ as markers, G β and G γ bands were excised and in-gel digested as described⁶², and then resolubilized in the presence of the “heavy labeled peptide cocktail” and run on a TSQ vantage triple quadrupole mass spectrometer (Thermo Scientific) following the scheduled multiple reaction monitoring method as described⁶². G β and G γ subunits were run separately and data were analyzed in Skyline. Correct peaks were manually chosen on the basis of retention time, dot plot values, relative distributions of transition ion, and heavy labeled peptide peaks. Samples were dropped from analysis if no correct peaks could be chosen. Furthermore, all chosen peaks with a signal-to-noise (S/N) ratio less than 5 for non-labeled peptides and 3.5 for labeled peptides were removed from further analysis. Using the light to heavy peak intensity ratio given in Skyline, the total volume of re-solubilized samples, injection volume, and the amount of heavy label peptide, G β and G γ subunits present in samples were calculated. Quantified G β and G γ subunits were then normalized by the total protein amount to account for variabilities between animals.

Validation of quantitative G β and G γ subunits MRM

G $\beta_1\gamma_2$ was serially diluted, separated by 12.5 % acrylamide SDS-PAGE, trypsin digested, mixed with heavy labeled peptide cocktail used in crude synaptosome studies, and run on TSQ with the quantitative MRM method as described in Fig. 2. The heavy labeled peptide was made with G β_1 amino acids 198–209, and 284–301, and G γ_2 amino acids 21–27 and 47–62. As described above, we calculate the G β_1 and G γ_2 detected per peptide using the light/heavy ratio in Skyline. The light to heavy ratio represents a ratio between the peak intensity of light and heavy proteotypic peptides. The ratio given in Skyline was timed with the amount of heavy label peptides and divided by the percent injection volume to calculate the amount of G β_1 or G γ_2 present in each sample tube. Then, the quantified G β_1 and G γ_2 by each proteotypic peptide (square or circle) were averaged and depicted as triangle in Fig. 2. The result was plotted in logarithmic scales and analyzed by linear regression to determine the limit of quantification and to assess the analytical reliability.

Statistical analysis

One-way analysis of variance (ANOVA) with a Tukey post hoc test was used to account for differences in protein expression of G β and G γ subunits (*, #, & p<0.05, **, ##, && P<0.01, ***, ###, &&& P<0.001). All statistical tests were performed using GraphPad Prism v. 7.0 for Windows, (GraphPad Software, La Jolla, California, USA, www.graphpad.com).

RESULTS

Development of the quantitative multiple reaction monitoring (MRM) method for G β and G γ subunits

To develop quantitative MRM method for neuronal G β and G γ subunits, we first selected two proteotypic peptides for four G β (G β_1 , G β_2 , G β_4 , and G β_5) and six G γ (G γ_2 , G γ_3 ,

$G\gamma_4$, $G\gamma_7$, $G\gamma_{12}$, and $G\gamma_{13}$) subunits from previous study⁶² and had heavy labeled versions of them synthesized (Table 1) (see Material and Method for more details). Non-neuronal $G\beta$ and $G\gamma$ subunits, such as $G\beta_3$, were not examined in this study. Two proteotypic peptides were selected per protein (see Material and Method for more detail) to increase the accuracy of quantification. No proteotypic peptides were selected for $G\gamma_5$ and $G\gamma_{11}$ as we were unable to identify a correct peak of sufficient signal intensity to pass acceptable signal to noise criteria in a previous study⁶².

To understand the detection intensities and fragmentation efficiency of each heavy labeled proteotypic peptide, for each protein, we performed scouting runs to determine appropriate amounts of heavy labeled proteotypic peptide (data not shown). As expected, each heavy labeled peptide had different fragmentation ion signal intensity. Based on these results and previous targeted mass spectrometry experiments⁶², we pooled appropriate concentration of heavy labeled peptides to make a heavy peptide cocktail that was digested with trypsin in the presence of BSA. This cocktail was added to all $G\beta$ and $G\gamma$ subunits samples after trypsin digestion and extraction from gel matrix. Using the light to heavy ratio calculated by the area under the curve⁶² of the sample and of heavy labeled peptides, we can quantify the protein level of neuronal $G\beta$ and $G\gamma$ subunits present in the sample (Fig. 1).

Validation of quantitative MRM of $G\beta$ and $G\gamma$ subunits

We next applied our method to examine serially diluted $G\beta_1\gamma_2$ (0.002 – 21.9 pmol) to determine the limit of detection, limit of quantification, and analytical accuracy of the method. Because $G\beta_1\gamma_2$ is the most abundant, easily expressed, and most commonly studied neuronal $G\beta\gamma$ dimer, we chose it as our standard for validation. We plotted the quantification value per peptide⁶⁷ and averaged (black) against with the fmol of $G\beta_1\gamma_2$ used. Here, we identified the limit of $G\beta_1$ and $G\gamma_2$ detection at 5.34 and 21.4 fmol of $G\beta_1\gamma_2$, respectively. Below the limit of detection, no peak passed the signal to noise test (see Material and Method for more details). The limit of $G\beta_1$ and $G\gamma_2$ quantifications were found at 85.5 and 42.7 fmol of $G\beta_1\gamma_2$ (Fig. 2). Because 42.7 fmol sample was excluded from the $G\beta_1$ quantification analysis, and 21.4 and 85.5 fmol samples had similar $G\beta_1$ level detected, we measured the limit of $G\beta_1$ quantification at 85.5 fmol (Fig. 2A). For all input quantities above the limit of quantification, we observed a linear increase in the amount of detected protein. Below the limit of quantification, the relationship between input and quantified protein was not linear. Using multiple proteotypic peptides for quantification has been shown to increase the confidence in quantification⁷⁴. Although we detect and quantify $G\gamma_2$ between 42.7 to 342 fmol using one heavy labeled proteotypic peptide, we are confident with the $G\gamma_2$ quantification in this range (Fig. 2B). Moreover, a linear regression test confirmed that the detected and quantified $G\beta_1$ and $G\gamma_2$ with two heavy labeled proteotypic peptides were proportional to the $G\beta_1\gamma_2$ input (Fig. 2). Based on the slopes of these curves, we determined that our method systematically over-estimates the amount of $G\beta_1$ by ~3 fold (Fig. 2A) whereas we under-estimate the amount of $G\gamma_2$ by ~90 fold (Fig. 2B). Although we attempted to provide a comprehensive absolute quantitative map, our data may not be absolute due to technical challenges associated with sample preparations. The presence of heavy labeled proteotypic peptides allows for quantification of non-labeled proteotypic peptides derived from proteolytic digestion of the target proteins. However,

several factors impact whether this quantification reflects an accurate measure of the absolute quantity of the target protein. Differences in peptide yield due to sample preparation artifacts and post-translational modifications along with differences in peptide resolubilization efficiencies and stability can lead to systematic errors in quantification⁷⁵. For example, the use of SDS in the postsynaptic fraction's resolubilization may affect the recovery and quantification of postsynaptic G γ subunits (see Discussion for more detail). Because of these, we are unable to infer absolute protein level of neuronal G β and G γ subunits, but we can accurately determine the expression pattern of neuronal G β and G γ subunits in any given sample.

The most abundant neuronal G β and G γ subunits in brain synaptosomes

Using our quantitative MRM method, we first determined the protein level of each G β and G γ subunit in crude synaptosomes (N=3) derived from whole mouse brain (Fig. 3). In ~13 mg of crude synaptosome lysate, we found G β_1 to be the most abundant neuronal G β subunit (Fig. 3A) supporting the previous finding⁷⁶. G β_4 and G β_5 , were the least abundant G β subunits in mouse crude synaptosomes (Fig. 3B). Moreover, G γ_2 might be the most abundant G γ subunits. G γ_{13} was the least abundant G γ subunit. G γ_2 was detected at 5.4 fold higher levels than G γ_{13} (Fig. 3C). In crude synaptosomes, the level of expression for G β subunits was G β_5 =G β_4 <G β_2 <G β_1 , and for G γ subunits was G γ_{13} =G γ_4 <G γ_7 <G γ_{12} <G γ_3 <G γ_2 . For the first time, we compared the protein level between each G β and G γ subunits. This is important for estimating the quantity of each G $\beta\gamma$ dimer *in vivo*. For example, G $\beta_1\gamma_2$ may be one of the most abundant G $\beta\gamma$ dimers *in vivo*. Its interactions with receptors and effectors may be more physiologically relevant than those between G $\beta_4\gamma_4$ or G $\beta_4\gamma_{13}$ and their effectors.

Subcellular localization of G β and G γ subunits

Because G $\beta\gamma$ dimers interact with a variety of effectors distributed throughout the cell, such as voltage gated calcium channels, potassium channels, and the SNARE complex^{9–16}, subcellular location of G $\beta\gamma$ dimers may also influence their effector selectivity. Here, we attempted to determine the subcellular localization of G β and G γ subunits (N=8, otherwise noted in Fig. 5). Crude synaptosomes of wildtype mice were separated into synaptosomal cytosolic, presynaptic, and postsynaptic fractions as described previously^{62, 69} (Fig. 4A). GAPDH was dominant in the synaptosomal cytosolic fraction. Syntaxin-1 and SNAP25 were dominant in a presynaptic fraction whereas postsynaptic density 95 (PSD-95) and NMDAR1 were dominant in a postsynaptic fraction (Fig. 4B), confirming the efficacy of our fractionation method. In these fractions, we examined the protein levels of each G β and G γ subunit. G β_1 was highly localized at the membrane, found in both pre- and post-synaptic fractions, whereas G β_2 was evenly distributed between all three fractions (Fig. 5A). Supporting the previous findings, both G β_4 and G β_5 were highly localized in the postsynaptic fraction (Fig. 5B)⁶². Overall, more G β subunits were detected in membrane fractions than the synaptosomal cytosolic fraction (Fig. 4B and 5).

G γ subunits also showed divergent localization patterns. The comparison was made between G γ subunits in each fraction (Fig. 5C–E) and between the synaptosomal cytosolic and presynaptic fractions (Fig. 5C–D) to account for the potential differences in resolubilization,

impacting the recovery and quantification of postsynaptic G γ subunits (see Discussion for more detail). In the synaptosomal cytosolic fraction, G γ_2 and G γ_3 seem to be abundant although no statistical significance was found (Fig. 5C). G γ_2 and G γ_3 may preferentially localize at the synaptosomal cytosolic fraction than the presynaptic fraction, $P=0.074$ and 0.076 (unpaired T-test) respectively. In the presynaptic fraction, G γ_{12} might be the most abundant G γ subunit while G γ_4 and G γ_{13} were the least abundant G γ subunits (Fig. 5D). Between the synaptosomal cytosolic and presynaptic fractions, G γ_7 showed no preference in a location, $p=0.866$. In the postsynaptic fraction, G γ_{12} , once again, might be the most abundant G γ subunits (Fig. 5E). Interestingly, G γ_4 and G γ_{13} were also the least abundant G γ subunits in the postsynaptic fraction (Fig. 5E). Although G γ_2 was one of the most abundant G γ subunits in the crude synaptosomes, it was not the most abundant G γ subunit in the presynaptic fraction where widely known G $\beta\gamma$ effectors are located.

Unlike G β subunits, more G γ subunits were found in the postsynaptic fraction than in the synaptosomal cytosolic fraction. Although we do not fully understand this phenomenon, we hypothesize that this may be due to the difference in re-solubilization buffers used for pre- and post-synaptic fractions as stated earlier (see Discussion for more detail).

DISCUSSION

Quantitative MRM of G β and G γ subunits

Because no dependable subunit-specific antibodies exist due to high sequence homology between subunits, studies of G β and G γ subunits at protein level have been limited. In this study, we present a method to perform a comprehensive, quantitative survey of G β and G γ subunits in crude synaptosomes. With heavy labeled proteotypic peptides cocktails, we quantified and compared the protein expression level of each G β and G γ subunit in whole and fractionated crude synaptosomes. Although our quantification method may only measure a subset of actual neuronal G β and G γ subunits present in crude synaptosomes due to technical challenges stated below, our method can now be applied to further understand and model how the expression and subcellular neuronal localization of G β and G γ subunits regulate dimerization and affect signaling pathways^{11, 62, 77}. As the sequences of G β and G γ subunits are highly conserved in mammals, the proteotypic peptides used in this method are conserved in human G β and G γ subunits, thus our method is suitable to evaluate G $\beta\gamma$ expression in human tissues.

Sample preparation, detection intensity, and fragmentation efficiency of each proteolytic peptide may impact the detected quantity of G β and G γ subunits. We enhanced the confidence in detection and quantification of all neuronal G β and G γ subunits by using two heavy labeled proteotypic peptides per protein. Non-neuronal G β and G γ subunits, such as G β_3 , and those with insufficient proteotypic peptides, such as G γ_5 , were not studied. Although G γ_5 was observed to be the most abundant G γ subunit in a neuronal cell line⁷⁸, we were not able to quantify G γ_5 . Further study will be needed to identify proteotypic peptides in regions that are not post-translationally modified and characterize the quantity and subcellular localization of G γ_5 in the brain. Each heavy labeled proteotypic peptide showed a different AUC demonstrating the importance of adjusting individual peptide amount for quantification (data not shown). Because we didn't know the amount of non-

labeled proteotypic peptides present in samples, we relied on previous data and detection of heavy labeled peptide alone to create an appropriate heavy labeled cocktail (data not shown)^{62, 74}. We were able to further adjust the heavy peptide cocktail for the subcellular localization study using the results of whole crude synaptosomes.

Purifying and creating validation curves for all neuronal G β and G γ subunits was impractical due to reagent limitations, so instead we used G $\beta_1\gamma_2$, which is the most widely used and physiologically relevant G $\beta\gamma$ dimer, to validate our quantitative MRM method (Fig. 2). As stated above, each proteotypic peptide has different physicochemical properties and is thus difficult to validate the quantification of neuronal G β and G γ subunits with a G $\beta_1\gamma_2$ standard alone. However, since all neuronal G β subunits and all neuronal G γ subunits were sampled together, we can extrapolate the experimental error of the quantification method. For example, any experimental manipulation affecting the proteotypic peptides of G β_1 likely affects the proteotypic peptides of the other neuronal G β subunits. Any systematic manipulation likely impacted all samples to a similar degree and thus accurately reflects the underlying expression pattern (Fig. 2). Instead of serial diluting the amount of heavy labeled G β_1 and G γ_2 proteotypic peptides, we also used the heavy peptide cocktail of the subcellular localization study to have a better estimate of quantification error relevant to our experimental design. Because our quantitative method depends on the peak intensity and peak intensity ratio of light and heavy proteotypic peptides, the experimental error may vary by the amount of heavy labeled proteotypic peptides present in the samples⁷⁵. It is important to predict the error with heavy labeled cocktail used in each experiment. Until we fully identify every G $\beta\gamma$ dimer present in the brain, we have determined that this is the best way to validate our quantitative method.

Overall, our data suggest that we under detect and quantify neuronal G γ subunits. The limit of G γ_2 detection was higher than G β_1 (Fig. 2B). We also calculated a higher error in absolute quantification of G γ_2 compared to G β_1 (Fig. 2B). Because G γ subunits are ~8 kDa, we may experience a greater loss of G γ than G β subunits in sample preparation and detection. It is known that G γ subunits are post-translationally modified, which may further complicate their detection. In validation experiments, the quantification of G γ_2 between 42.7 to 342 fmol was done using one heavy labeled proteotypic peptide because the 2nd peptide didn't pass the signal to noise criteria (Fig. 2B). A previous study⁶² showed that this peptide has higher signal intensity than the other peptide in lending us confidence in the G γ_2 quantification in this range (Fig. 2B). Despite these technical challenges, our quantitative MRM method is a valid and reliable method to quantify and compare the different protein levels of neuronal G β and G γ subunits.

Expression and subcellular localization of G β subunits in brain synaptosomes

Similarly to previous studies, we determined that G β_1 was the most abundant G β subunit in whole crude synaptosomes^{21, 42, 62}. The expression of G β_1 was 3.5 fold more than G β_2 and ~130 fold more than G β_4 and G β_5 (Fig. 3A and B) supporting a critical role for G β_1 in G β subunit signaling in processes in the brain. G β_1 is involved in neural development; its knockout leads to perinatal lethality with reduced cortical thickness, brain volume, and impaired neural progenitor cell proliferation⁷⁶. Mutations in G β_1 are also found in patients

with severe neurodevelopmental disability, hypotonia, and seizures³³. Compared to Gβ₁, significantly fewer Gβ₄ and Gβ₅ are available for dimerization in the brain (Fig. 3B). Taken the result of previous study⁶² in tandem with our quantitative data, Gβ₅ is the least abundant Gβ subunit present only in the striatum.

Our results may provide a partial rationale for Gβγ selectivity for effectors because Gβγ dimers containing Gβ₁, concentrated in presynaptic and postsynaptic fractions (Fig. 5A), may preferentially activate membrane bound effectors such as VDCC compared to Gβγ dimers containing Gβ₂. Dimers with Gβ₁ strongly inhibit VDCC activity⁷⁹ compared to Gβ₂ and may affect VDCC activity in pain, Parkinson's disease, and epilepsy⁸⁰. Moreover, Gβ₄ and Gβ₅ were largely restricted to postsynaptic fractions suggesting that dimers with these subunits may primarily interact with postsynaptic effectors. While postsynaptic effectors of Gβγ dimers containing Gβ₄ remain unknown, Gβ₅ was shown to specifically interact with the Gγ-like domain of regulator of G protein signaling (RGS)7 family proteins instead of Gγ subunits^{42, 81–88}. These RGS 7 family proteins were found to affect dopamine signaling in striatum for motor learning and locomotor responses⁸³, supporting the phenotype of Gβ₅ knockout mice^{89, 90}. Significant loss of RGS 7 family members were also found in Gβ₅ knockout mice⁹⁰. Because RGS9-2 - Gβ₅ complex was found at the membrane, predominantly at the postsynaptic fraction^{85, 91}, whereas RGS7-Gβ₅ complex was found in the cytosolic fraction^{83–85, 88}, further studies will be needed to elucidate the *in vivo* presence of RGS9-2-Gβ₅ complex over RGS7-Gβ₅. In addition, further studies with various Gγ subunits will be needed to understand and verify the *in vivo* ramifications of these Gβ subunit differences in Gβγ localization.

Expression and subcellular localization of Gγ subunits in brain synaptosomes

We determined that Gγ₂ was one of the most abundant Gγ subunits in whole crude synaptosomes, supporting previous studies^{21, 62}. In addition to Gγ₂, Gγ₃, and Gγ₁₂ were the most abundant in whole crude synaptosomes. Together with our Gβ result, this may support the hypothesis that Gβ₁γ₂ is one of the most abundant dimers present in whole crude synaptosomes. Although the *in vivo* role of Gβ₁γ₂ has not been fully characterized, we speculate that its abundant expression may make Gβ₁γ₂ vital to a wide range of signaling pathways. Based on our quantitative results, we have gained further insight into the quantity and location of the following Gβγ dimers *in vivo*. Because significantly less Gγ₄ and Gγ₁₃ were detected in whole crude synaptosomes, Gβ₄γ₄ and Gβ₄γ₁₃ may be the least abundant Gβγ dimers. Although Gβ₁ and Gβ₂ are known to interact with Gγ₄^{45, 92}, the low abundance of Gγ₄ disfavors the formation of Gβ₁γ₄ and Gβ₂γ₄ dimers.

In subcellular localization study, we overall detected a higher level of Gγ subunits in the postsynaptic fraction than presynaptic fraction. We speculate that this may be due to the presence of SDS in the re-solubilization buffer of the postsynaptic fraction pellet. In presence of 0.5% SDS, which has higher critical micelle concentration than Triton X-100^{93, 94} in the re-solubilization buffer, we may be enhancing the recovery of Gγ subunits in the postsynaptic fraction. To account for this, the comparison was made between Gγ subunits in each fraction (Fig. 5C–E) and between the synaptosomal cytosolic and

presynaptic fractions only (Fig. 5C–D). In both synaptosomal cytosolic and presynaptic fractions, 1% Triton X-100 was used.

Although no statistical significant differences were found within the synaptosomal cytosolic fraction and between the synaptosomal cytosolic and presynaptic fractions, $G\gamma_2$ and $G\gamma_3$ seem to be localized at the synaptosomal cytosolic fraction (Fig. 5C). Of these $G\gamma$ subunits, $G\gamma_{12}$ was the only $G\gamma$ subunit that was more likely to be abundant in the presynaptic fraction than the synaptosomal cytosolic fraction ($p=0.092$). Interestingly, a recent epigenetic study found increased $G\gamma_{12}$ expression in cigarette smokers compared to non-smokers⁹⁵, which may link $G\gamma_{12}$ to nicotinic cholinergic signaling. $G\gamma_{12}$ abundance at the membrane may also be influenced by the phosphorylation of $G\gamma_{12}$ by protein kinase C^{38, 60}, which anchors it to the membrane by enhancing its interaction with $G\alpha$ subunits and the adenosine A_1 receptor^{58, 60, 96}. Phosphorylation of $G\gamma_{12}$ was not monitored in this study; therefore, further studies will be needed to determine if $G\gamma_{12}$ phosphorylation is required for its abundance at the membrane. In addition to $G\gamma_{12}$, $G\gamma_3$ and $G\gamma_7$ might be the most abundant $G\gamma$ subunits in the presynaptic fraction (Fig. 5D) while $G\gamma_2$ and $G\gamma_3$ might be in the postsynaptic fraction (Fig. 5E). Unlike $G\gamma_3$ and $G\gamma_{12}$ that were abundant in both fractions, $G\gamma_7$ may preferentially interact with presynaptic effectors. Similar to the whole crude synaptosomes' data, $G\gamma_4$ and $G\gamma_{13}$ were the least abundant $G\gamma$ subunits in both membrane fractions (Fig. 5 E).

Together with $G\beta$ subunit data, we can further speculate which $G\beta\gamma$ dimers may be present in each fraction. For example, $G\beta_1\gamma_2$ and $G\beta_1\gamma_3$ are more likely to be present in the synaptosomal cytosolic fraction than in presynaptic fraction. Because $G\gamma_3$ preferentially interacts with $G\beta_1$ and $G\beta_2$, $G\beta_1\gamma_3$ and $G\beta_2\gamma_3$ may be the dominant dimers in the synaptosomal cytosolic fraction⁵⁰. Within the pre- and postsynaptic fractions, $G\beta_1\gamma_{12}$ may be one of the most abundant dimers. Further *in vivo* study will be needed to verify the quantity of each dimer stated above.

$G\beta\gamma$ selectivity for GPCRs and effectors

To date, very little is known about the *in vivo* selectivity of $G\beta\gamma$ dimers for particular GPCRs and effector proteins. Various *in vitro* studies hint at possible $G\beta\gamma$ preferences^{25–29, 51}. For example, $G\beta_1\gamma_3$ may associate with somatostatin GPCRs while $G\beta_3\gamma_4$ dimers interact with M4 muscarinic GPCRs to inhibit L-type Ca^{2+} channel^{28, 97}. An increased susceptibility to seizures in $G\gamma_3$ knockout mice may be due to the loss of activity mediated by somatostatin GPCRs^{50, 98}. In addition, $G\beta_2\gamma_2$ interacts with the galanin receptor to inhibit a Ca^{2+} channel⁹⁹. The wide difference in expression patterns within subcellular fractions could reflect the unique contribution of each $G\beta$ and $G\gamma$ subunit to different signaling pathways⁶². However, further functional *in vitro* and *in vivo* studies will be required to validate the presence of particular $G\beta\gamma$ dimers and their selectivity for certain GPCRs and effectors. As our current method can only detect specific $G\beta$ and $G\gamma$ subunits separately, further method development is needed to identify proteotypic peptides of each $G\beta\gamma$ dimer as a whole. Taking advantage of transgenic mice with cell-type specific promoters and HA or FLAG tagged GPCRs, and overexpression of viral vectors expressing

particular G $\beta\gamma$ dimers, we hope to further elucidate G $\beta\gamma$ selectivity for GPCRs and effectors in future studies.

CONCLUSIONS

Here, we addressed the protein level and subcellular localization of neuronal G β and G γ subunits using the quantitative MRM mass spectrometry in whole and fractionated crude synaptosomes. Our study supports the previous findings that G β_1 is the most abundant G β subunit while G γ_2 is one of the most abundant G γ subunits in whole crude synaptosomes. G β_1 was mostly localized at the membrane while G β_2 was evenly distributed throughout the membrane and synaptosomal cytosolic fractions. In addition to G γ_2 , G γ_3 and G γ_{12} were also abundant in whole crude synaptosomes. G γ_3 and G γ_{12} were abundant in both pre- and post-synaptic fractions. We highlight the *in vivo* distribution of neuronal G β and G γ subunits using a new quantitative tool and thus provide insights into the G $\beta\gamma$ dimers assembly in normal brain function. Although further efforts will be necessary to further improve the quantification strategies and evaluate the selectivity of G β and G γ subunits to form G $\beta\gamma$ dimers, this work represents an important advance in the field of GPCR signaling, especially the selectivity of G $\beta\gamma$ dimers formation. Because the expression, localization, and affinity of the G β and G γ subunits have been hypothesized to affect G $\beta\gamma$ dimerization and G $\beta\gamma$ -effector interaction, our study will have a far reaching impact for those interested in the regulatory effects of G $\beta\gamma$ dimer specificity not only in physiology but also in disease pathology.

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Figure 1. The workflow of quantitative multiple reaction monitoring (MRM) experiment

Lysed whole or fractionated synaptosomes from wild-type adult mice were run on an SDS-PAGE gel to separate G β and G γ subunits. Then, G β and G γ subunits bands were excised, digested with trypsin, and resuspended with the heavy peptide cocktail (red star). Samples were injected to HPLC connected to triple quadrupole mass spectrometry with the quantitative MRM method. The ratio of non-labeled proteotypic peptide (light) and heavy labeled proteotypic peptide was used to calculate the amount of G β and G γ subunits present in samples. See result and experimental design sections for the detail descriptions.

Figure 2. Quantification of $G\beta_1$ and $G\gamma_2$

To validate the quantitative MRM of $G\beta$ and $G\gamma$ subunits, 14 samples containing serial dilution of purified $G\beta_1\gamma_2$ dimer were examined for the quantification of $G\beta_1$ (A) and $G\gamma_2$ (B). After the signal to noise test, 13 and 11 samples were quantified for $G\beta_1$ (A) and $G\gamma_2$ (B), respectively. The subunit $G\beta_1$ (A) and $G\gamma_2$ (B) were quantified based on $G\beta_1$ 198-209 (circle in A) and 284-301 (square in A) and $G\gamma_2$ 21-27 (circle in B) and 47-62 (square in B). These two proteotypic peptides per subunits were averaged and indicated as triangles (See Material and Method for more details). For $G\beta_1$, R^2 were 0.995 ($G\beta_1$ 198-209) and 0.997 ($G\beta_1$ 284-301). For $G\gamma_2$, R^2 were 0.982 ($G\gamma_2$ 21-27) and 0.999 ($G\gamma_2$ 47-62). The

linear range represents the analytical reliability of the quantitative MRM method (R^2 of averaged = 0.996 (**A**) and 0.998 (**B**)).

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Figure 3. The protein level of Gβ and Gγ subunits in whole crude synaptosomes
 Quantification of Gβ subunits (**A and B**) and Gγ subunits (**C**) in ~13 milligrams (mg) of lysate (N=3). Sub-view at 1–10,000 fmol (**B**). Gβ1 was the most abundant Gβ subunit (P=0.040 compared to Gβ2, and 0.007 compared to Gβ4 and Gβ5) when Gγ2 might be the most dominant Gγ subunit (P=0.050 compared to Gγ7, and 0.008 compared to Gγ4 and Gγ13). Data were presented as mean ± SEM and compared by a one-way ANOVA, *P<0.05, **P<0.01. *Post hoc* analysis was performed with Tukey's multiple comparison test.

Figure 4. Subcellular fractionation of whole crude synaptosomes
The workflow of fractionation experimental protocol (**A**), and representative western blot of fractionated samples (**B**). GAPDH was dominant in a synaptosomal cytosolic fraction. Syntaxin-1 and SNAP25 were dominant in a presynaptic fraction when postsynaptic density 95 (PSD-95) and NMDAR1 were dominant in a postsynaptic fraction. Gβ₁₋₄ were detected in synaptosomal cytosolic, pre-, and post-synaptic (PSD) fractions but concentrated at the membrane fractions.

Figure 5. Subcellular localization of Gβ and Gγ subunits

Quantification of Gβ subunit per mg of protein lysate (**A and B**) (N=8, otherwise noted).

Sub-view at 1–3,000 fmol (**B**). (**C–E**) Quantification of Gγ per mg of protein lysate in synaptosomal cytosolic (**C**), presynaptic (**D**), and postsynaptic fractions (**E**) (N=8, otherwise noted). Data were presented as mean ± SEM and compared by a one-way ANOVA, *, # P<0.05, **, ##P<0.01, ***, ###P<0.001. *Post hoc* analysis of Tukey's multiple comparison test was performed. Statistical significances were found compared to synaptosomal cytosolic * and presynaptic# fractions (**A and B**), and Gγ₁₂ (**C–E**).

Table 1

List of heavy labeled proteotypic peptides

Name	Sequence position	Peptide Sequence	Precursor m/z	charge	Product ion m/z
Gβ₁	198–209 284–301	(R)LFVSGACDASA K (L) (R)JLLAGYDDFNCNVWDAL K (A)	617.3048 1068.0215	2 2	487.23, 787.35, 874.38, 973.45 640.35, 898.40, 954.94, 1127.54
Gβ₂	198–209 257–280	(R)TFVSGACDASI K (L) (R)ADQELLMYSHDNIICGTSVAFS R (S)	632.3101 917.4432 / 922.7749	2 3	508.25, 829.40, 916.43, 1015.50 419.23, 490.26, 676.37, 777.41, 949.52
Gβ₄	198–209 305–314	(R)TFVSGACDASS K (L) (R)SGVLACHDN R (V)	619.2841 518.2639 / 345.8450	2 2 / 3	495.23, 675.29, 803.34, 890.38, 989.44 340.16, 396.70, 446.24, 474.75, 608.28, 679.31, 792.40
Gβ₅	129–139 318–327	(K)VIVWDSFTTN K (E) (R)VSILFGHEN R (V)	659.3501 591.3187	2 2	705.37, 820.39, 1006.47, 1105.54 498.27, 622.29, 769.36, 882.45
Gγ₂	21–27	(K)MEANID R (I)	429.7043 / 437.7018	2	527.28, 598.32, 727.36
Gγ₃	47–62 3–17	(K)EDPLITPVASENPF K (E) (K)GETPVNSTMSGQA K (K)	896.4612 779.3819 / 787.3793	2 2	774.43, 927.46, 1123.58, 1224.62 441.24, 554.33, 635.83, 643.82, 686.35, 694.35, 960.48, 1074.52, 1090.52
Gγ₄	25–31 3–17	(K)JEASLC R (I) (K)EGMSNNSTTSISQA R (K)	429.7225 796.8641 / 804.8615	2 2	458.24, 545.27, 616.31, 745.35 471.25, 584.34, 671.37, 873.47, 960.50
Gγ₇	51–66 19–25	(R)EDPLIIPVASENPF R (E) (R)JEAGIE R (I)	902.4794 399.2232	2 2	927.46, 1123.58, 1236.66 484.28, 555.31, 684.36
Gγ₁₂	45–60 5–15	(R)NDPLLGVVASENPF K (D) (K)TASTNSIAQA R (R)	852.9560 565.2954	2 2	738.42, 897.46, 1053.55, 1152.61 655.38, 769.42, 957.50
Gγ₁₂	23–29 18–23	(R)LEASIE R (I) (K)YQLAF K (R)	414.2285 389.2229	2 2	514.29, 585.32, 714.37 373.23, 486.32, 614.38
	37–44	(K)WIEDGIP K (D)	483.2627	2	252.18, 422.29, 537.31, 666.35

Red highlighted arginine and lysine residues were heavy labeled by ¹³C or ¹⁵N. Bolded sequence position indicates proteotypic peptides with the strongest fragmentation ion intensity. These peptides were used for the heavy labeled proteotypic peptides detection analysis (data not shown).